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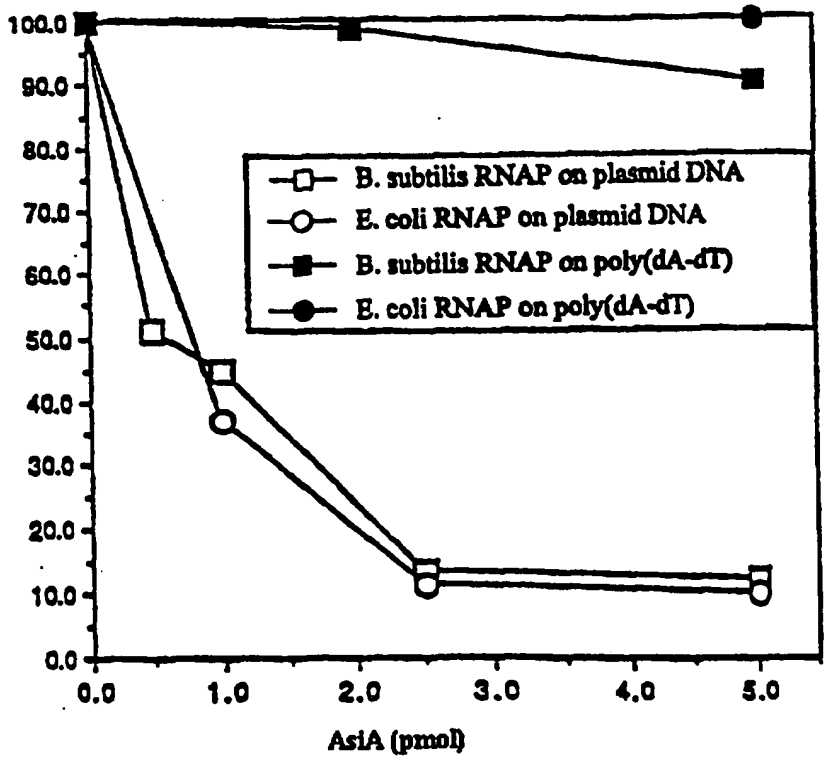
(54) Title: METHOD FOR TREATMENT OF DISEASE WITH ANTI-SIGMA FACTOR AsiA

(57) Abstract

A method for treating diseases caused by infection by a bacterial pathogen is provided. The method comprises administering to patients in need thereof a therapeutically effective amount of the AsiA protein.



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METHOD FOR TREATMENT OF DISEASE WITH ANTI-SIGMA FACTOR AsiA

FIELD OF THE INVENTION

This invention relates to a method for treating a variety of diseases. Specifically, it relates to a method for treating infection by a bacterial pathogen. The invention is premised on the discovery that the T4 bacteriophage anti-sigma factor AsiA protein interacts with a highly conserved region of the sigma 70 subunit of *Escherichia coli* (*E. coli*) RNA polymerase and is lethal to the organism.

BACKGROUND OF THE INVENTION

RNA polymerase (RNAP) is an enzyme that catalyzes the synthesis of an RNA strand from either a DNA or an RNA template. Unless stated otherwise, however, this term typically refers specifically to DNA-dependent RNA polymerase, which is responsible for the transcription of RNA from a DNA template. RNA polymerase of bacteria has a very large complicated subunit structure. The active form of the enzyme, referred to as the holoenzyme, contains at least four different subunits in the ratio ($\alpha_2\beta\beta'\sigma$). Additional subunits may be associated with the enzyme depending on the species of bacteria. The catalytic site of RNA polymerase is contained in the $\alpha_2\beta\beta'$ portion of the enzyme, which is referred to as the core enzyme. The σ subunit (σ factor) is a regulatory subunit that enables the core enzyme to recognize promoter sites, thereby initiating transcription.

Bacteria have one or two major sigma factors and several minor sigma factors. The major sigma factor of the organism is responsible for most RNA synthesis in the cell and is essential for cell survival. The minor sigma factors can be divided into two groups. One group, such as heat shock proteins, are specific σ factors that have evolved to direct transcription at specific promoters. The second group of minor σ factors are closely related to the major σ factors. but these σ factors are nonessential for cell growth. (*See* Lonetto *et al.* (1992) J. Bacteriol.

174:3843-3849). Inhibition of a minor σ factor would generally not be fatal to the organism and in fact, as discussed below, inhibition of minor σ factors appears to be a common regulatory device used by organisms.

Studies have revealed that the major σ factors of all bacteria, both Gram-positive and Gram-negative, are very similar, having an amino acid sequence homology of at least 51%. (Gribskov and Burgess (1986) Nucleic Acids Res. 14:6745-6763; Helmann and Chamberlin (1988) Annu. Rev. Biochem. 57:839-872; Lonetto *et al.* (1992) J. Bacteriol. 174:3843-3849). Four regions of high conservation have been identified. Specifically, regions 1.2, 2.1, 2.2, 2.3, 2.4, 3.1, 3.2, 4.1, and 4.2 are all highly conserved. (Lonetto *et al.* (1992) J. Bacteriol. 174:3843-3849).

Bacteriophage T4 (T4), a lytic enterobacteria whose principle host is *Escherichia coli* (*E. coli*), has been the subject of extensive biochemical studies. The genome of T4 is linear double stranded DNA. Transcription of the T4 DNA occurs in three main stages: early, middle and late. Each stage occurs at a distinct time after infection and is initiated at a distinct class of promoters.

Initiation at T4 early promoters requires no T4-coded proteins. Shortly after infection a 90 amino acid, 10 kDa protein, referred to herein as the AsiA protein, is synthesized by T4. The AsiA protein is coded for by an early T4 gene, referred to herein as the *asiA* gene. The isolation, cloning and sequencing of this gene and the partial purification of the AsiA protein are described in Orisini *et al.* (1993) J. Bacteriol. 175:85-93. (*See* Table 1).

The AsiA protein binds to the sigma 70 subunit of *E. coli* and inhibits the interaction of the *E. coli* RNA polymerase core enzyme with its σ^{70} subunit. (Stevens (1972) Proc. Nat. Acad. Sci. USA 69:603-607; Stevens (1974) Biochemistry 13:493-503; Stevens (1975) Biochemistry 14:5074-5079; Stevens (1976) in RNA Polymerase, eds. Losick and Chamberlin (Cold Spring Harbor Laboratory, Cold Spring, NY) pp.617-627; Stevens (1977) Biochim. Biophys. Acta 475:193-196). *E. coli* is a Gram positive bacterium and the σ^{70} subunit is its major σ factor.

Recent studies by Brody and coworkers have shown that the AsiA protein

inhibits recognition of early T4 promoters, thereby shutting off early T4 transcription. They have also shown that the AsiA protein cooperates with another T4 phage protein, MotA, to activate middle mode T4 transcription. It is also believed that AsiA plays a role in the transition from middle to late transcription. (*See* Orisini *et al.* (1993) J. Bacteriol. 175:85-93; Ouhammouch *et al.* (1994) J. Bateriol. 176:3956-3965; Ouhammouch *et al.* (1995) Proc. Natl. Acad. Sci. 92:__). All of these functions are presumably related to the interaction of the AsiA protein with the σ^{70} subunit of *E. coli.*

The amount of AsiA protein synthesized in T4 infected cells appears to be very strictly controlled. First, the ribosome binding site, GUGG is very poor which constitutes a first limit on the amount of AsiA protein produced. (Stormo *et al.* (1982) Nucleic Acids Res. 10:2997-3011). Second, like many early genes, *asiA* gene RNA synthesis diminishes around 3-4 minutes after infection at 30 °C. It has been shown that the *asiA* gene early promoter (P158.7) shares extended homologies with other T4 early promoters, all of which seem to undergo the same transcriptional shut-off. (Leibig *et al.* (1989) J. Mol. Biol. 208:517-536). As discussed above, recent research by Brody and coworkers has shown that the AsiA protein itself is directly involved in shutting-off early transcription. Thus, the AsiA protein shuts off transcription of its own mRNA. Finally, the *asiA* mRNA contains an internal GGAG sequence that is cut by the T4 RegB endoribonuclease, further limiting the amount of AsiA protein in infected cells. (Uzan *et al.* (1988) Proc. Natl. Acad. Sci. USA 85:8895-8899; Ruckman *et al.* (1989) New Biol. 1:54-65).

The existence of a σ factor binding protein that inhibits σ activity is not unique to T4-infected *E. coli* cells. The *Salmonella typhimurium flgM* gene product has been shown to bind and inhibit the activity of the *S. typhimurium* fagellum-specific σ factor, σ^F (Ohnishi, *et al.* (1992) Mol. Microbiol. 6:3149-3157). The anti-σ factor SpoIIAB, is part of a regulatory circuit that has been shown to bind to and inhibit transcription initiation factor σ^F in the gram-positive soil bacterium *Bacillus subtilis (B. subtilis).* (Duncan and Losick (1993) Proc. Natl. Acad. Sci. USA 90:2525-2329; Min *et al.* (1993) Cell 74:735-742 (σ^F is a

sporulation sigma factor in *B. subtilis*)). Benson and Haldenwang (Proc. Natl. Acad. Sci. USA 90:2330-2334 (1993), have provided evidence for a similar regulatory circuit for the control of σ^B, the secondary σ factor of *B. subtilis*. In this system, the anti-σ factor RsbW binds to σ^B and inhibits its association with core RNA polymerase.

All of the anti-sigma factors identified to date, however, have been shown to inhibit minor sigma factors only, which may or may not have conserved sequences. No other anti-sigma factors have been identified that inhibit the major sigma factor of an organism. Because the major sigma factors are highly conserved among all prokaryotes, an anti-sigma factor which inhibits a major sigma factor, in a conserved region, would likely be useful as a broad spectrum antibiotic. Additionally, such an antibiotic would not be toxic to humans, because the RNA polymerases of humans are quite different from the RNA polymerases of bacteria. There is no homology between the conserved sequences of the major sigma factors in bacteria and any RNA polymerase.

SUMMARY OF THE INVENTION

The present invention describes a method for the treatment of infection by a bacterial pathogen by administering the T4 anti-sigma factor protein, AsiA to patients in need thereof. The invention is premised on the discovery that the T4 bacteriophage anti-sigma factor AsiA interacts with a highly conserved region of the sigma 70 subunit of *Escherichia coli* (*E. coli*) RNA polymerase and that any expression from a cloned AsiA gene efficiently kills *E. coli*.

The inventors further show that the T4 bacteriophage anti-sigma factor AsiA also inhibits the RNA polymerase activity of *Bacillus subtilis (B. subtilis)*, a gram positive bacteria and *Mycobacterium smegmatis* (*M. smegmatis)*, a gram negative bacteria that is closely related to *Mycobacterium tuberculosis* (*M. tuberculosis*).

Because these three bacteria are so far apart on the evolutionary scale, logic dictates that the AsiA protein is acting on a very highly conserved, discrete region of the major sigma factors. The conclusion to be drawn from this is that the AsiA

protein will inhibit all microorganisms which carry this conserved region in their major sigma factors.

Finally, this invention includes a method for the purification of the AsiA protein.

BRIEF DESCRIPTION OF THE FIGURES

FIGURE 1 illustrates the inhibition of the sigma- dependent plasmid DNA transcription in both *E. coli* (O) and *B. subtilis* (□) by varying concentrations of the AsiA protein. Figure 1 also illustrates that sigma-independent poly(dA-dT) transcription, in both *E. coli* (○) and *B. subtilis* (□), is unaffected by the presence of the AsiA protein.

FIGURE 2 illustrates the inhibition of the sigma- dependent plasmid DNA transcription in both *B. subtilis* (□) and *M. smegmatis* (O) by varying concentrations of the AsiA protein. Figure 2 also illustrates that sigma-independent poly(dA-dT) transcription, in both *B. subtilis* (□) and *M. smegmatis* (○) was unaffected by the presence of the AsiA protein.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention relates to methods for treating diseases caused by bacterial pathogens. The method comprises the administration of a therapeutically effective amount of the AsiA protein to a patent suffering from infection caused by a bacterial pathogen. Example 1 describes the expression and a reproducible method of purification of the AsiA protein. A standard strain used for the expression of foreign proteins in *E. coli* is BL21 (DE3). The foreign protein is cloned under control of a T7 late promoter. The T7 late RNA polymerase (gene 1) is in this strain under control of a *lac* promoter. This strain also contains *lac* repressor, which represses synthesis of gene 1 *m*RNA. Adding isopropyl-β-D-thiogalactopyranoside (IPTG) induces the T7 late polymerase, which in turn synthesizes the *m*RNA of the foreign protein. The T4 AsiA gene is unclonable in this system. This result is not surprising, in that, genes for a number

of toxic proteins cannot be synthesized in this way. The reason for this is that the *lac* repressor does not repress 100% of the *m*RNA synthesis from the *lac* promoter. Therefore, there is a small amount of RNA synthesized from gene 1 (this phenomenon is referred to as leaky RNA synthesis). This leaky RNA synthesis of gene 1 RNA produces enough protein to induce the toxic protein. If one uses a strain that carries a pAIQ7, which overproduces *lac* repressor, then one can get clones carrying the *asiA* gene, which is proof that this is the correct explanation for the inability to clone the *asiA* using the standard technology. Even the clones produced in this manner, however, are not stable, and the cloned *asiA* gene tends to be lost (selected against) during growth.

Because of this problem a different system is used to clone the *asiA* gene under T7 late promoter control (Example 1). The cloning is done with *E. coli* JM101 and the T7 gene 1 product is brought into the cell by λ CE6, a λ phage derivative which expresses the T7 gene 1 protein after infection. Even in this case, in which there is no gene 1 protein in the cell, the cloned *asiA* gene which carries the wild type Shine-Dalgrano sequence, GUGG, is stable, but an engineered gene carrying a strong Shine-Dalgrano sequence, GGAGG, tends to accumulate insertion sequence (IS) elements in the cloned *asiA* gene. This necessitates frequent re-isolation of a correct *asiA* gene when cloning this plasmid. The conclusion to be drawn from this is that even very minute quantities of AsiA protein are toxic to *E. coli.* This explains why the production of AsiA is so highly regulated by the T4 phage.

The purified protein was then used to measure the ability of AsiA to inhibit *B. subtilis* and *M. smegmatis* (Examples 2 and 3). *B. subtilis* is a Gram positive bacteria. Its major sigma factor is sigma A (σ^{43}). (Lonetto *et al.* (1992) J. Bacteriol. 174:3843-3849). *M smegmatis* is a Gram positive bacterium, a mycobacterium which is closely related to *M. tuberculosis*, the bacteria which causes tuberculosis. Mycobacteria are important pathogens.

Figure 1 illustrates that the inhibition of RNA polymerase activity by the AsiA protein is restricted to sigma dependent RNA synthesis and does not affect sigma independent transcription (poly dA-dT transcription). This result suggests

that the AsiA protein binds to one of the highly conserved motifs of the major sigma factors. Figure 1 also shows that the inactivation of the RNA polymerase from *B. subtilis* follows the same dose response curve as that of the inactivation of the RNA polymerase from *E. coli.* This result further indicates that the site of binding by the AsiA protein is conserved between major sigma factors of Gram negative (*E. coli* σ^{70}) and Gram positive (*B. subtilis* σ^{A}) bacteria.

Figure 2 shows that AsiA also inhibits the sigma A dependent RNA synthesis of *Mycobacterium Smegmatis*. *M. Smegmatis* is a gram positive, mycobacterium, which is closely related to *M. tuberculosis*, a casual agent of tuberculosis.

As discussed above, because these three bacteria are so far apart on the evolutionary scale, logic dictates that the AsiA protein is acting on a very highly conserved, discrete region of the major sigma factor. The conclusion to be drawn from this is that the AsiA protein will inhibit and be toxic to all microorganisms which carry this conserved region in their major sigma factors. Microorganisms of particular interest are *Staphylococcus aureus (S. aureus)*, a gram positive bacteria, which is a major human pathogen, *Pseudomonas*, a genus of Gram negative bacteria and *M. tuberculosis*, the bacteria which causes tuberculosis.

The mode of administration of the materials described herein include intravenous, intraperitoneal, and intramuscular injections, as well as all of the other standard methods for administering therapeutic agents to a subject. These methods are standard and will be evident to those skilled in the art.

The AsiA protein may be too large to penetrate bacterial cell walls. Although not limited by theory there are several possible means for administration, if this is in fact the case. One possibility for the administration of the AsiA protein as a broad spectrum antibiotic will be to cut down the size of this protein, in order to determine the minimal peptide sequence necessary to retain biological activity. Peptide antibiotics, such as polymyxins and bacitracin, do exist. Polymyxins are a group of peptide antibiotics whose molecular weight is between 1000-1200. Bacitracin is a cyclic dodecapeptide antibiotic.

A second possibility is to administer the protein together with low doses of

a second antibiotic, which inhibits cell wall synthesis. By inhibiting cell wall synthesis the second antibiotic would permeabilize the bacteria sufficiently to allow the 10.5 kDa AsiA protein to penetrate and kill the bacteria.

Finally, the Asia-σ^{70} complex will be studied by X-ray crystallography and multiple dimensional NMR in order to determine the structure of the complex. Once this has been accomplished, a small molecule which has the same contacts and geometry of contacts as AsiA will be synthesized. This molecule should also be effective as a broad spectrum antibiotic and be capable of administration by standard means. This approach to the development of antibiotics is becoming standard in the industry.

The following examples are provided for illustrative purposes only and are not intended to limit the scope of the invention.

EXAMPLE 1. EXPRESSION AND PURIFICATION OF THE ASIA PROTEIN.

E. coli JM101 bacteria carrying the plasmid pBAS-M1 were grown and infected as previously described. (Orsini *et al.* (1993) J. Bacteriol 175:85-93). The pBAS-MI plasmid carries a copy of the T4 *asiA* gene with an improved ribosome binding site, under the control of a T7 promoter (Orsini *et al.* (1993) J. Bacteriol 175:85-93). After lysis, proteins were subject to $(NH_4)_2SO_4$ fractionation. The bulk of AsiA was soluble in 50% saturated $(NH_4)_2SO_4$. It was then fractionated on a Sephacryl S-100 column. AsiA-containing fractions were pooled and the KCl concentration was adjusted to 50 mM before loading on a Q-Sepharose Fast Flow anion exchange column (Pharmacia), which had been equilibrated with TGED/50 mM KCl [TGED: 50 mM Tris-HCl (pH 7.9), 0.1 mM EDTA, 0.1 mM dithioreitol (DTT), 5% glycerol]. A 50 - 500 mM KCl linear gradient in TGED was used to elute AsiA. AsiA eluted as a homogeneous peak at about 275 mM KCl.

The purified AsiA protein was used in the following experiments to measure its ability to inhibit sigma-dependent and -independent transcription by the RNA polymerase of *E. coli, B. subtilis* and *M. smegmatis*.

EXAMPLE 2. Inhibition of Sigma-dependent Transcription in *E. Coli* and *B. Subtilis*.

To test the ability of the RNA polymerase of *E. coli* and *B. Subtilis* to initiate transcription of a specific gene in the presence of AsiA, plasmid DNA, containing the *B. Subtilis trp* E promoter (pU*trp* E, generously provided by Dr. Paul Gollnick) was linearized with *Hind* III, and used as a template for the *in vitro* run-off transcriptions. Transcriptions were carried out with 0.5 pmol of DNA, 1 pmol of RNA polymerase holoenzyme, in the presence of 100 mM KCl, 10 mM $MgCl_2$, 40 mM Tris-HCl, pH 7.9, 0.1 mM EDTA, 0.1 mM DTT, 0.4 mM ATP, 0.4 mM GTP, 0.4 mM CTP, 0.1 mM UTP and 5 mCi of [^{32}P-α]-UTP. Also included was 10 μg of acetylated BSA in a 50 μl reaction. The results of the inhibition experiment are shown in Figure 1. The number of pmols of pure AsiA protein is indicated on the abscissa. The amount of trp E run off transcript synthesized in a 10 minute reaction at 37 °C was quantified by microdensitometry and is indicated on the ordinate. For *E. coli* RNA polymerase, the concentration of KCl was 190 mM. Control experiments were done with 2 μg of poly(dA-dT) as the template in a 100 μl reaction mixture. Incorporation of [^{3}H-α]-UTP into trichloroacetic acid insoluble material was measured for each point.

As can be seen in Figure 1 the inactivation of the RNA polymerase from *B. subtilis* follows the same dose response curve as that of the inactivation of the RNA polymerase from *E. coli*. Additionally, inhibition of RNA polymerase activity by the AsiA protein is restricted to sigma dependent RNA synthesis and does not affect sigma independent poly dA-dT transcription.

EXAMPLE 3. Inhibition of Sigma-dependent Transcription in *B. Subtilis* and *M. Smegmatis*.

To test the ability of the RNA polymerase of *B. Subtilis* and *M. Smegmatis* to initiate transcription of a specific gene in the presence of AsiA, the heterologous *B. subtilis sin* P3 promoter in plasmid pIS109 was used as a template. This plasmid, obtained by cloning of the Xba-HindIII fragment of pIS109 (Guar *et al.* (1986) J. Bacteriol. 168:860-869) into similarly digested

pUC19, has two terminators downstream of *sinR* (Guar *et al.* (1988) J. Bacteriol. 170:1046-1053). The HindIII site, located downstream of these terminators, was used to linearize the plasmid for *in-vitro* run-off transcriptions. Transcription reactions were carried out with 1 μg of DNA, 1 pmol of RNA polymerase holoenzyme, in the presence of 10 mM $MgCl_2$, 40 mM Tris-HCl pH 7.9, 0.1 mM EDTA, 0.1 mM DTT, 0.4 mM ATP, 0.4 mM GTP, 0.4 mM CTP, 0.1 mM UTP and 5 mCi of [^{32}P-α]-UTP. Also included was 5 μg of acetylated BSA in a 50 μl reaction. The results of the inhibition experiment are shown in Figure 2. The number of pmols of pure AsiA protein is indicated on the abscissa. The amount of pIS109 run off transcript synthesized in a 7 minute reaction at 37 °C was quantified using a PhosphorImager, and is indicated on the ordinate. For the *B. subtilis* RNA polymerase, the concentration of KCl was 100 mM. The reactions with *M. smegmatis* polymerase contained 10% glycerol. Control experiments were done with 2 μg of poly(dA-dT) as the template in a 100 μl reaction mixture. Incorporation of [^{3}H-α]-UTP into trichloroacetic acid insoluble material was measured for each point.

As can be seen in Figure 2 the inactivation of the RNA polymerase from *M. smegmatis* follows the same dose response curve as that of the inactivation of the RNA polymerase from *B. subtilis*. Additionally, inhibition of RNA polymerase activity by the AsiA protein is restricted to sigma dependent RNA synthesis and does not affect poly dA-dT transcription.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: BRODY ET AL.

(ii) TITLE OF INVENTION: Method for Treatment of Disease with Anti-sigma Factor AsiA

(iii) NUMBER OF SEQUENCES: 1

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(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Diskette, 3 1/5 inch, 1.44 MG storage

(B) COMPUTER: IBM compatible

(C) OPERATING SYSTEM: MS-DOS

(D) SOFTWARE: WordPerfect 6.0

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

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(B) FILING DATE: 15 FEBRUARY 1995

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(C) REFERENCE/DOCKET NUMBER: NEX26/PCT

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 400 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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CLAIMS

1. A method for treating diseases caused by bacterial pathogens comprising administering to a patient in need thereof a therapeutically effective amount of the T4 anti-sigma factor AsiA protein.

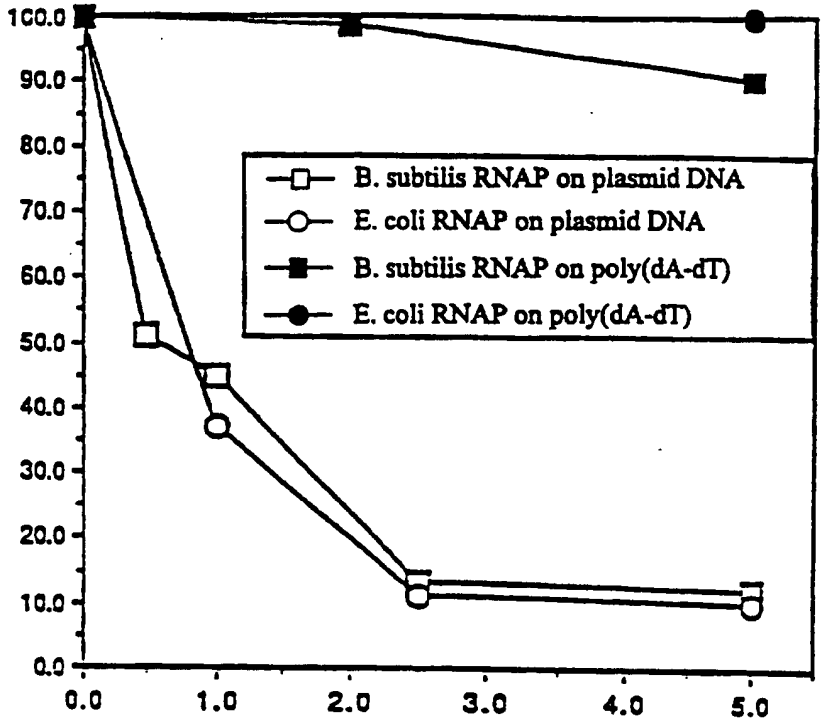
2. The method of claim 1 wherein the bacterial pathogen is Gram positive or Gram negative.

3. The method of claim 1 wherein said bacterial pathogen is *Staphylococcus aureus*.

4. The method of claim 1 wherein said bacterial pathogen is a *Pseudomona*.

5. The method of claim 1 wherein the bacterial pathogen is a mycobacterium.

6. The method of claim 3 wherein said mycobacterium is *Mycobacterium tuberculosis* .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20.0
10.0
0.0
0.0
1.0
2.0
3.0
4.0
5.0
B. subtilis RNAP on plasmid DNA
E. coli RNAP on plasmid DNA
B. subtilis RNAP on poly(dA-dT)
E. coli RNAP on poly(dA-dT)
AsiA (pmol)

FIG. 1

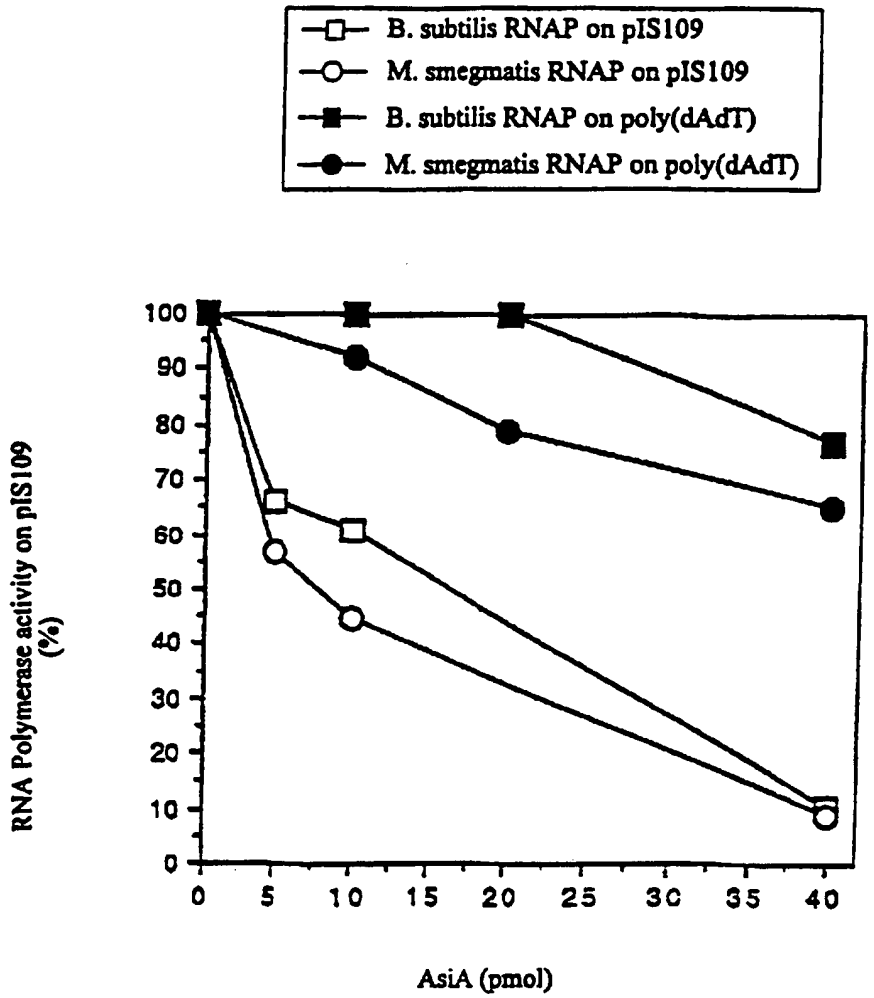
B. subtilis RNAP on pIS109
M. smegmatis RNAP on pIS109
B. subtilis RNAP on poly(dAdT)
M. smegmatis RNAP on poly(dAdT)
RNA Polymerase activity on pIS109 (%)
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
35
40
AsiA (pmol)

FIG. 2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/00642

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 38/00
US CL :514/12
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/12

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

none

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Journal of Bateriology. Volume 175, Number 1, issued January 1993, Orsini et al., "The AsiA Gene of Bacteriophage T4 Codes for the Anti-sigma 70 Protein.", pp. 85-93, especially page 89, Overexpression of gene AsiA, first line.	1-6
Y	Journal of Bateriology, Volume 174, Number 12, issued 1992, Lonetto et al., "The sigma 70 family: Sequence Conservation and Evolutionary Relationships ", pp. 3843-3849, especially p. 3843, second column, Group I. Primary Sigma Factors, lines 3-5.	1-6

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 25 MARCH 1996	Date of mailing of the international search report 05 APR 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer M. Delacroix Telephone No. (703) 308-0196

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